ANTI-ARTHRITIC POTENTIAL OF STANDARDIZED EXTRACT OF *CLERODENDRUM SERRATUM* (L.) : A TRADITIONAL MEDICINAL PLANT

by

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SYNOPSIS

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INTRODUCTION

Introduction to Plant

World plant biodiversity is the largest source of herbal medicine and still about 60 –80 % world population relies on plant based medicines which are being used since the ancient ages as traditional health care system. Plants are biosynthesizing large amount of secondary metabolites through various metabolic pathways. These are very complex and having unique carbon skeleton structures and many of them are of high interest to the pharmaceutical and chemical industry. An enormous number of compounds are undergoing pre-clinical and clinical trials in the last decades, especially in the area of diabetes, cancer, malaria etc. WHO says in current situation major limitation for merchandising benefit of herbal drugs is standardization. Standardization of the plant is mandatory as backed by all the regulatory authorities of world like FDA, MHRA, CDCSCO.

Clerodendrum serratum belonging to the family Verbenaceae an Ayurvedic plant to Indian system which is rich in metabolites. It is a deciduous shrub widely distributed in Western ghats[1]. In Ayurveda, the plant is well known as Bharangi (Sanskrit) and commonly known as Blue Glory (English). As per the traditional claims the root is the potential source of drug for ailments such as asthma, body ache, bronchitis, cholera, dropsy, eye diseases, fever, inflammations, malaria, opthalmia, rheumatism, snakebite, tuberculosis, ulcers and wounds[2]. Phytoconstituents present in the root part of this plants are apigenin-7-glucoside, D-mannitol, γ-sitosterol and ursolic acid[2].

Introduction to Arthritis

Arthritis is a chronic inflammatory disease/disorder characterized by inflammation of the synovial membrane, pain and restricted joint movement. The pathogenesis of arthritis are not completely understood. An external trigger (e.g., cigarette smoking, infection, or trauma) that triggers an autoimmune reaction, leading to synovial hypertrophy and chronic joint inflammation along with the potential for extra-articular manifestations[3].

Synovial cell hyperplasia and endothelial cell activation are early events in the pathologic process that progresses to uncontrolled inflammation and consequent cartilage and bone destruction. Genetic factors and immune system abnormalities contribute to disease propagation.

CD4 T cells, mononuclear phagocytes, fibroblasts, osteoclasts, and neutrophils play major cellular roles in the pathophysiology of arthritis. Abnormal production of numerous cytokines, chemokines, and other inflammatory mediators eg, tumor necrosis factor alpha (TNF-α), interleukin IL-1, IL-6, transforming growth mainly fibroblast growth factor (FGF) has been demonstrated in patients with arthritis[4].

The interaction of genetic and environmental factors results in a cascade of immune reactions, which ultimately lead to the development of synovitis, joint damage, and structural bone damage. These, in turn, lead to pain, disability, and emotional, social and economic challenges. A number of extraarticular manifestations and comorbidities are present in patients with rheumatoid arthritis (RA), which result in increased mortality[5].

Epidemiologic data indicate that the occurrence of arthritis and related diseases will have a significant impact upon the practice of pharmacy[6-14]. Explanatory style was associated with an increased risk of developing RA and increased mortality rate in patients with RA[15]. The number of RA sufferers is increasing, which is probably a result of increasing life expectancy[16]. The risk of arthritis increases with age and arthritis is more common among women than men. From 2013- 2015, an estimated 54.4 million US adults (22.7%) had some form of arthritis like RA, gout, lupus, or fibromyalgia annually. Of persons ages 65 or older, 49.6% ever reported doctor-diagnosed arthritis[17]. Arthritis affects 15% people i.e. over 180 million people in India.

Non-steroidal anti-inflammatory drugs (NSAIDs) are often prescribed to patients with rheumatoid arthritis (RA), as the first line drugs and are administered over a long term. NSAIDs is classified principally into acidic and basic preparations, and the former is more widely used for the treatment, since the latter doesn’t possesses anti-rheumatic effect[18]. The other mode of treatment are use of disease-modifying anti-rheumatic drugs (DMARDs) and corticosteroids (steroids). Above mention existing synthetic drugs have several limitations due to extra-articular manifestations or co-morbidities. Among these gastrointestinal bleeding, cardiovascular problems are the major adverse effect associated with chronic arthritis. Moreover, the prevalence is higher than many well known diseases such as diabetes, AIDS and cancer.

Natural products have been an important basis for the development of new pharmaceuticals and has a reservoir of lead structure. The first Indian National Health Policy 1983 claims that India is the richest source of herbs and the drugs should be standardized. World-wide

usage of medicinal plants depends upon the standardization of individual plant. Moreover, WHO guidelines also strictly advise the standardization of medicinal plants for their safety and efficacy. With this focus, current work is designed to standardize the *Clerodendrum serratum* extract and evaluate its biological activity as anti-arthritic and to provide scientific explanation for traditional use of *Clerodendrum serratum* as an anti-arthritic agent.

AIMS AND OBJECTIVES

The current study has the following aims and objectives:-

1. Phytochemical analysis and standardization of *Clerodendrum serratum* extract
2. HPLC method validation of plant extract, according to ICH Guidelines
3. Evaluation of Anti-arthritic activity

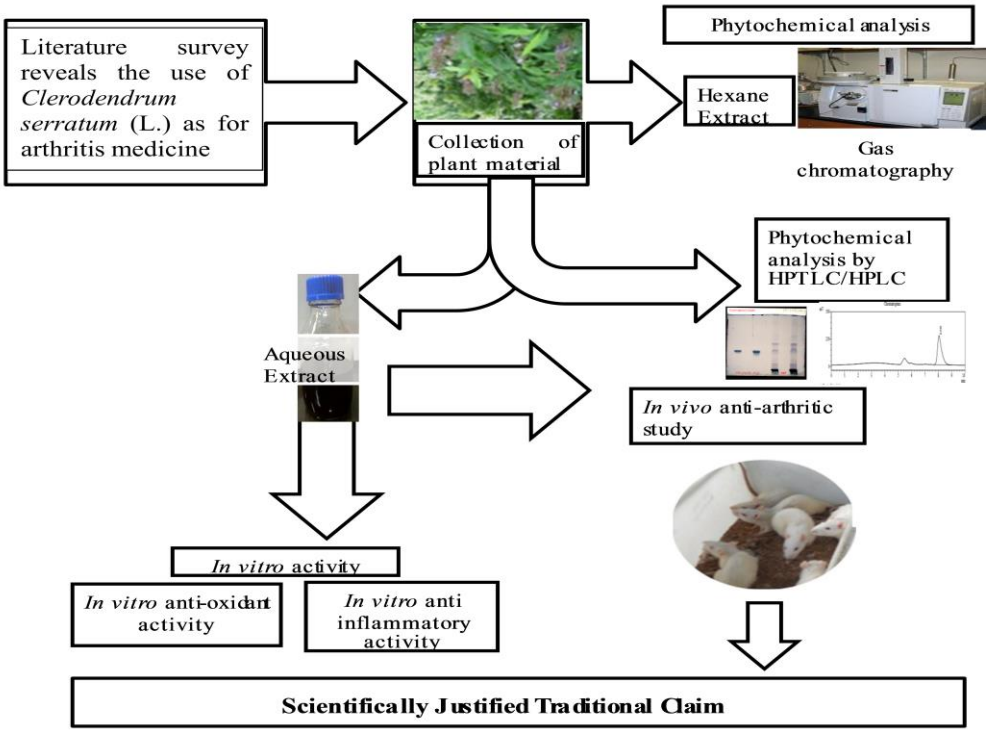
Literature survey reveals the use of Clerodendrum serratum (L.) as for arthritis medicine
Collection of plant material
Phytochemical analysis
Hexane Extract
Gas chromatography
Phytochemical analysis by HPTLC/HPLC
Aqueous Extract
In vivo anti-arthritic study
In vitro activity
In vitro anti-oxidant activity
In vitro anti inflammatory activity
Scientifically Justified Traditional Claim

Figure 1: Overview of the study

Phytochemical analysis and standardization of *Clerodendrum serratum* extract

Collection and authentication of plant material

Roots of *Clerodendrum serratum* L. (Verbenaceae) was purchased from the local market of Faizabad Uttar Pradesh, India. The taxonomical recognition was fulfilled, from the Department of Agronomy, Aacharya Narendra Dev Agriculture Technical University, Dist. Faizabad, Uttar Pradesh.

Preparation of Extract

The collected brown colored roots (100gm) were extracted (decoction) including water (500ml) at a temperature not exceeding 110°C for 2hrs. The extract was allowed to cool, filtered and lyophilized. The yield obtained for dried extract was 10%w/w.

Qualitative Analysis of extract

The phytochemical screening of aqueous extract was concluded to identify the secondary metabolites (table 1) present in the aqueous extract of *C. serratum* by different chemical reactions[19-21].

Table 1: Secondary metabolites present in aqueous extract of *Clerodendrum serratum*

Sl. No.	Secondary Metabolites	Result	Sl.No.	Secondary Metabolites	Result
1	Glycoside	Present	4	Volatile oil	Present
2	Alkaloid	Present	5	Tannins	Present
3	Sterols	Present	6	Triterpenoids	Present

Quantitative analysis of phytochemicals by spectrophotometer

Total phenolic content, total flavonoid content, saponin content and alkaloid content were fixed by using distinct chemicals/reagents expressed in (Table 2). All the chemicals were analytical grade and attained from Sigma Aldrich Co., St. Louis, USA. Quantification of the secondary metabolites was realized using spectrophotometer[19, 22].

Qualitative HPTLC study of Ursolic acid

High performance thin layer chromatography (HPTLC) was executed to confirm the presence of ursolic acid in the aqueous extract (figure 2). Solvent system, toluene: ethyl acetate (8:2 v/v) was adopted to standardize the analyte. Visualizing reagent, anisaldehyde sulfuric acid was passed

down to confirm the spots. A numeral compounds were visualized in the chromatogram of aqueous extract of *Clerodendrum serratum.* Plant extract exhibit the presence of ursolic acid compared with that of standard ursolic standard (R_f 0.38).

Table 2: Secondary metabolites present in aqueous extract of *Clerodendrum serratum*

Total Flavonoid content in mg/g equivalent to Rutin	
Sample	67.67±3.51
Total Alkaloid content in mg/g equivalent of Atropine	
Sample	12.67±3.78
Total Saponin content in mg/g equivalent of Diosgenin	
Sample	38.2±1.31
Total Phenolic content in mg/g equivalent of Gallic acid	
Sample	134.4±0.91

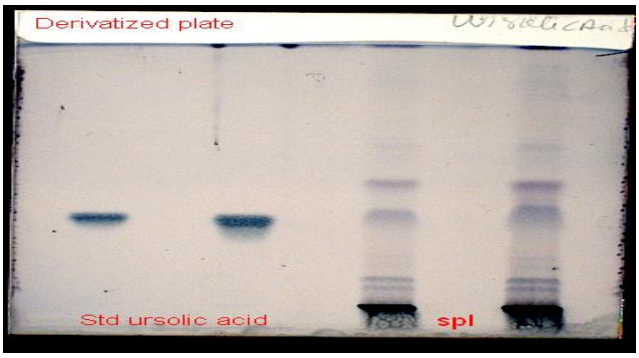

Figure 2 : HPTLC chromatogram of *Clerodendrum serratum* extract and ursolic standard

Phytochemical analysis of volatile principles

The phytoconstituents present in the n-hexane root extract was evaluated by gas chromatography–mass spectrometry using Clarus-500 Mass Spectrometer. Temperature programmed with a ramp 8°C /Min upto 240°C and ramp 6°C /Min upto 280°C hold for 15 min while the injector temperature kept at 280°C at the injection volume of 1µL with a syringe size of 10µL. The Flow rate was maintained at 1.2ml/min. The Oven was sustained for 70 min having a transfer line temperature for about 280°C. Scanning was done in the range of 35-500 amu. Identification of phytoconstituents was contrived by comparison of their mass spectra with NIST libraries mass spectra.

GC/MS chromatograms of n-hexane root extract of *C. serratum* L. showed several peaks indicating the presence of a wide range of volatile principles. A total of 15 constituents was

identified and characterized from the root of *C. serratum* L. Components were identified by using the combination of retention index value and mass spectral matching against library standards.

HPLC method validation of plant extract, according to ICH Guidelines

Preparation of Reference Solution: 10 mg of ursolic acid working standard was taken into 10 ml volumetric flask. 5 ml of methanol was combined and sonicated. The solution was settled to room temperature, dilution was done up to the mark with methanol and mixed. Further dilution was done in a subsequent volumetric flask by taking 3 ml of solution diluted with methanol.

Preparation of Sample Solution: 1.5 gm of test sample was appropriated in Iodine flask and 25 ml of water was added followed by sonication for proper mixing. Reflux was done for 30 min in reflux assembly. The process was repeated twice and the filtrate was evaporated to dryness. Obtained residue was dissolved in methanol and blended with 10 ml methanol. About 0.5 ml of this solution was mixed with methanol in 10 ml volumetric flask.

Methanol and acetonitrile in the ratio of 30:70 (v/v) was used as mobile phase. HPLC of the aqueous extract showed the presence of ursolic acid in the test sample as compared to standard. A plot between the concentration of a standard solution of ursolic acid and peak response gave a straight line with a correlation coefficient less than 0.998. The recovery of the extract, i.e. 95%-110% for the standards were obtained when spiked with standard at four different concentration levels. The HPLC method was established to give repeatable results at different concentrations and thereby the method was found to be reliable. The peak purity of the sample was evaluated by comparing retention time, peak area of test sample and standard. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 33µg/ml and 0.059% for the ursolic acid was determined from the regression equation. Validation of the method for ursolic acid was done according to ICH guidelines for linearity, precision, accuracy, range, specificity and ruggedness.

Acceptance criteria RSD<2.5.

Acceptance criteria RSD<5 for Ruggedness.

Evaluation of Anti-arthritic activity

Antioxidant activity was performed using DPPH, Superoxide scavenging assay and Ferric reducing antioxidant power (FRAP) assay.

DPPH assay: The DPPH assay of the extract was determined by using UV-Spectrophotometer [23-25] which involved the preparation of standard, test and control. Different solutions of the extract were prepared in methanol to give concentrations in the range of 20 - 100µg/ml. 1.5 ml of each extract solution was blended with 200 µM DPPH solution, followed by incubation around 30 min. Optical density was measured after 30 min against methanol (as blank) at 517 nm.

Superoxide scavenging assay: 1 ml of alkaline DMSO contains 0.1 ml of 5mM NaOH and 0.9 ml of DMSO. NBT (Nitroblue Tetrazolium) solution was processed by defronting 25 mg of nitroblue tetrazolium in 25 ml DMSO to give a concentration of 1 mg/ml. Different concentration (20 - 100µg/ml) of rutin and test sample was prepared. To the reaction mixture, carrying 0.1 ml of NBT, 0.3 ml of aqueous extract and 1 ml of alkaline DMSO was added, adjusting volume to 1.4 ml and the absorbance was fixed at 560 nm. Plain DMSO used as blank and the reaction mixture without extract (water inspite of extract) used as control. Depreciate absorbance of the reaction mixture designated the increasing of superoxide anion scavenging activity. 50% inhibition of extract was determined by plotting a graph between absorbance and concentrations[26].

Ferric reducing antioxidant power (FRAP) assay: The endowment to reduce ferric ions was deliberated using the method as described below [27-29]. Rutin and test samples at divergent combinations (100 to 500 µg/ml) was supplemented to 3 ml of FRAP reagent and the counterpoise was nurtured at 37°C for 30 min. The rise in absorbance at 593 nm was measured. The antioxidant capacity is based upon the ability to reduce ferric ions of sample to ferrous ions.

The IC_{50} values were found to be 85.43 µg/ml and 107.59 µg/ml for DPPH radical scavenging and Superoxide scavenging assay respectively, whereas FRAP showed significant reducing power activity with increased concentration of the sample. The study showed a significant correlation existed between concentrations of the extract and the percentage engrossment of free radicals.

In-vitro Anti inflammatory Activity: Hypotonic solution–Induced haemolysis or membrane stabilizing activity and Effect of Protein Denaturation.

Hypotonic solution –Induced haemolysis or membrane stabilizing activity:

The test sample comprised of stock erythrocyte (RBC) suspension (0.03ml) infused with 5ml of hypotonic solution (154mM NaCl in 10mM Sodium Phosphate Buffer at pH 7.4) ranging from

concentration 100-500 µg/ml. The control sample consisted of 0.03ml RBC suspension with a hypotonic buffered solution. The standard drug acetylsalicylic was treated similarly. The experiment was done in triplicate. The mixtures were allowed for incubation for 10 minutes at room temperature, followed by centrifugation for 10 minutes at 3000rpm and absorbance of the supernatant was uniformed spectrophotometrically at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was computed by following equation tabulated in table 3[30-32].

$$\% \text{ Inhibition of haemolysis} = 100^* [A1 – A\ 2\ /A\ 1]$$

Where:

A 1 = Absorbance of hypotonic buffered solution alone

A 2 = Absorbance of test /standard sample in hypotonic solution.

Table 3: % Inhibition of test sample with standard

S. No.	Conc. (µg/ml)	% Inhibition	
		Standard ASA	Sample RKTCAS
1.	100	37.93±0.247	8.67±0.276
2.	200	42.97±0.310	12.40±0.310
3.	300	47.14±0.117	16.95±0.276
4.	400	51.48±0.117	20.76±0.193
5.	500	57.23±0.089	22.90±0.234

Effect of Protein Denaturation[33-34]: Test solution consisting of 1ml of different concentrations of test sample ranging from 100-500 µg/ml and standard acetylsalicylic acid 100-500 µg/ml was composite with 1ml of egg albumin solution (1mM) and incubated at 27 ±1˚C for 15 minutes. Denaturation was allowed by keeping the reaction confection at 70˚C in a water bath for 10 minutes. After cooling the turbidity was measured spectrophotometrically at 660 nm. Percentage inhibition of denaturation was calculated from control without sample and standard. All the experiment was performed in triplicate tabulated in table 4.

Table 4: % Inhibition of test sample with standard

S. No.	Conc. (µg/ml)	% Inhibition	
		Standard ASA	Sample RKTCAS
1.	100	67.73±0.448	39.62±0.448
2.	200	73.49±0.356	44.25±0.625
3.	300	79.89±0.178	47.98±0.448
4.	400	82.80±0.205	51.66±0.719
5.	500	85.23±0. 178	55.93±0.544

In- vivo anti arthritic study

Complete Freund's adjuvant (CFA) induced arthritis model was used to evaluate the anti arthritic activity. Albino Wistar male rats of weight 200±25 gm were used for the experiment. The dose adopted was 100mg/kg and 200mg/kg b.w. Paw diameter, joint diameter, arthritic score, body weight was used as a parameter for the activity. Estimation of TNF-α and COX-2 was also done. Animals were divided into five groups. Thirty minutes after oral administration of the vehicle/drug, 0.1 ml of CFA (0.05% Mycobacterium butyricum in mineral oil) was injected into the subplantar surface of the left hind paw with a 26 gauge needle. Paw diameter, joint diameter, arthritic score, body weight measurements was carried out on days 3rd, 7th, 14th and 21st. On day 21st, the animals were sacrificed and terminal blood collection was done. The serum was separated and TNF-α, COX-2 levels were estimated by ELISA kit assay method. The ankle joints were collected and stored in formalin for histology. All were compared to standard Indomethacin and evaluated.

C. serratum showed progressive decreased in paw diameter for CFA induced as 4.89mm and 4.43mm for aqueous extract at dose levels of 100mg/kg and 200mg/kg, respectively as compared to standard 4.17mm for Indomethacin at 21st day. Moreover swelling in joint diameter was significantly reduced to 7.62mm and 7.10mm for 100mg/kg and 200mg/kg respectively in comparison to Indomethacin i.e. 6.65mm. Arthritic score for 200mg/kg b.w. was 1.67 and whereas in case of standard Indomethacin i.e. 1.33. A significant body weight gain was observed as compared to disease control animals. Level of TNF-α and COX-2 were 8.40ng/ml and 7.14ng/ml for 200mg/kg body weight respectively whereas 5.03ng/ml and 6.40 for standard.

Summary of the research work

India being one of the 12 mega bio-diversity, culturally firm and have strong knowledge about the use of plants and people live in their own style in different communities. Keeping this in mind the present study was carried out to assess the richness of ethno-medicinal plant species with a proper scientific approach, used by the different communities for arthritis disorders.

Roots of *Clerodendrum serratum* L. (Verbenaceae) was purchased from the local market of Faizabad Uttar Pradesh, India. The taxonomical recognition was done from Department of Agronomy, Aacharya Narendra Dev Agriculture Technical University, Dist. Faizabad, Uttar Pradesh.

The collected brown colored roots (100gm) were extracted (decoction) with water (500ml) at a temperature not exceeding 110°C for 2hrs. The extract was allowed to cool, filtered and lyophilized. The yield of the dried extract was 10%w/w.

Phytochemical analysis of aqueous root extract indicated the presence of triterpenoids, volatile oil, sterols, alkaloid and glycoside. Both the HPTLC and HPLC analysis reveals the presence of ursolic acid. Further GC-MS analysis of hexane root extract indicated the presence of 15 phytoconstituents. The root extract was standardized by HPLC and the method was validated as per ICH guidelines. The validated HPLC method is established for ursolic acid. Quantitative estimation revealed 0.059% ursolic acid present in the extract determined from the regression equation. The HPLC method was validated for the quantitative estimation of ursolic acid from *Clerodendrum serratum* extracts.

The proposed HPLC method provides a quantitative estimation of ursolic acid in raw material and extracts of *Clerodendrum serratum*. The method is accurate, precise, linear, rugged, specific, simple, rapid and within the range. Hence the present HPLC method is suitable for the quality control of the raw materials, extracts and assay of the markers in *Clerodendrum serratum*.

The aqueous extract of the root was subjected to *in vitro* and *in vivo* anti arthritic activity. The extract showed IC_{50} values around 85.43 µg/ml and 107.59 µg/ml for DPPH radical scavenging and Superoxide scavenging assay, respectively, whereas FRAP showed significant reducing power activity with increased concentration of the sample.

The extract showed around 8% and 40% inhibition for membrane stabilizing and protein denaturation with 100 µg/ml concentration. The results of the present experiments indicate that

Clerodendrum serratum possesses significant anti-arthritic activity. The possible mode of anti-arthritic activity of aqueous extract of *Clerodendrum serratum* may be mediated mainly by inhibition of COX-2 and TNF-α as seen in various parameters like paw edema, arthritic index and joint diameter. On the basis of results, anti-arthritic activity may be due to shielding of synovial membrane, obstruction of cartilage destruction and inhibition of vascular permeability. Moreover, the results obtained in the current study shows favorable response of the plant from restoration of arthritis in terms of body weight, arthritic score, paw edema, joint diameter.

In summary, the current study established the scientific basis of ethnomedicinal use of *Clerodendrum serratum* for arthritis. The study also reveals the medicinal importance of the plant as an anti-inflammatory and free radical scavenger. The plant has potential to be commercialized in the future. The study also documents the use and significance of *Clerodendrum serratum* as ethnomedicinal plant and also suggest a possible alternate solution for arthritis to overcome limitation and drawbacks of current medicines.

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